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Forensic biological fluids : mixed sample evaluations and light degradation

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FORENSIC BIOLOGICAL FLUIDS: MIXED SAMPLE EVALUATIONS AND LIGHT DEGRADATION

A Thesis

Presented to

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Abstract

This study addressed two issues dealing with forensic human identification using STR loci. The first aspect of the study evaluated the ratio at which a minor contributor in a mixture ceases to be detected. This study evaluated mixtures prepared with semen, blood, and saliva in ratios that range from 25:1 to 1:1. The second portion attempted to determine if ultraviolet light, sunlight, and fluorescent light had any degradative effects on biological fluids. Blood and semen were exposed for varying time increments and evaluated for degradation. The minor component in a mixture was reliably detected even when its concentration was 1/25 of the major component. Blood samples were reliably typed at nine STR loci for samples up to 13 months old, under all light conditions. Semen samples exposed to sunlight and fluorescent light were reliably typed after 13 months. Some degradation was observed with semen sampled exposed to ultraviolet light.

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Background and Significance

Forensic human identification methods rely on testing DNA from cells in epithelium, blood and sperm. DNA analysis may be used to solve questions ranging from paternity to identifying a serial rapist. Parents contribute a portion of their DNA to an evolving zygote, thereby determining the genetic profile of an individual. Each parent contributes one allele for a particular chromosomal locus to the child. The combination of the maternal allele and the paternal allele comprises an individual's genotype at that particular locus. It is this genotype, or genetic profile, that is the basis for forensic human identification.

Alleles may exist as a sequence polymorphism or as a variable number of repeats of a core sequence. This research focuses on the second case, specifically short tandem repeat (STR) sequences. The variability in the number of times a core sequence is repeated characterizes an individual. The number of repeat units in a row, or tandem repeats, is highly variable, allowing for powerful discrimination between individuals (1,2,3). This characterization makes STR DNA analysis a reliable method for making statistically compelling human identifications and for distinguishing one individual from another (4,5).

Separation of the DNA from impurities in a biological sample is accomplished with a simple chloroform/phenol extraction. Once the DNA is extracted and is present in a suitable solution, the DNA may be used to determine a person's genotype at various

genetic loci. However, the DNA must first be copied or amplified thousands of times so that it is present in elevated concentrations for analysis.

The polymerase chain reaction (PCR) is the amplification process by which a specific region of a chromosome is copied in an exponential fashion. This amplification is achieved by first identifying a region of interest which possesses the potential for variability. Once this region is identified, assuming it has already been sequenced, a set of primers is created which recognizes the nucleotide sequence upstream from the region of interest. These primers initiate a DNA synthesis reaction that copies the region of interest. The other regions of the chromosome in question, as well as all other chromosomes, are reduced to negligible amounts due to the specificity of the primers.

Cutting edge technology in the area of PCR includes the use of short tandem repeat units. STRs are regions that contain a repeat sequence ranging from two to six nucleotides in length. It has been demonstrated that the best results for forensic samples are obtained with repeat units of at least four base pairs in length (6). These tetrameric STRs are highly polymorphic, found throughout the human genome, and form the basis for forensic STR testing (2). Slipped strand mispairing, or "stutter", occurs much less frequently than with repeat units of two or three base pairs (1), which makes interpretation much easier. For this reason, the two base pair and three base pair loci are not used in forensic science applications (7). STR alleles are inherited in a predictable manner (2) and can be typed in multiplexed and automated reactions (4). That is, several primer sets may be mixed together with the DNA and dNTPs, allowing for amplification of up to ten loci in a single reaction. Thus, one gains the amplified products of all ten

loci in the same time that it takes to amplify at one locus. In addition, multiplexing reduces the amount of forensic sample consumed during the amplification process.

PCR based systems are capable of providing a more distinctive profile of an individual (4). Previously, most analyses were restricted to enzyme polymorphisms or restriction fragment length polymorphisms (RFLP). RFLP methods, while more discriminating than conventional enzyme polymorphisms, present their own challenges. Conventional protein electrophoresis requires that the enzyme be in good condition. RFLP requires a large amount of high molecular weight DNA (8,9). RFLP and protein electrophoresis are both time consuming processes, requiring anywhere from several days to several weeks for results to be obtained. PCR systems are adept at typing degraded samples or samples with low input DNA concentrations and do so in a more timely fashion than RFLP (8). The first PCR marker to be used in forensic applications was HLA DQα, which has six alleles. While this system was an improvement over protein electrophoresis and RFLP, scientists soon demanded a more discriminating system (9). A second marker, D1S80, which has 28 alleles, was later added to the PCR repertoire. While D1S80 improved upon HLA DQα in terms of discrimination power, the analysis process was time consuming and prone to preferential amplification problems (9). The AmpF*l*STR Profiler Plus™ kit (Perkin-Elmer, Foster City, CA), with its nine loci and sex determination locus, provides greater discrimination power than DQα and D1S80 combined. In addition to increased discrimination power, STR analysis is a sensitive and accurate technique that produces results in a short period of time (8).

For the purposes of this project, the AmpF*l*STR Profiler Plus™ kit (Perkin-Elmer, Foster City, CA) was used, which contains the following loci in a single multiplex amplification reaction: D3S1358, vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820 and Amelogenin. A table detailing the chromosome location, common sequence motif, size range in base pairs, and dye label may be found in Table 1.

Currently, one of the more popular means of STR DNA analysis uses capillary electrophoresis with fluorescent detection. Capillary electrophoresis allows for multiplexing of different loci as well as high throughput of samples (9). This is achieved by adding a fluorescent tag to one primer of each primer set present in the multiplex amplification reaction (1). The fluorescent dye tags are assigned to a particular set of primers so that loci with overlapping size ranges may be simultaneously detected by the instrument, allowing for real-time result evaluation. The fluorescent dyes that are typically used are ROX™ (red), 5-FAM™ (blue), JOE™ (green) and NED™ (yellow). The internal sizing standard, which adjusts for size migrations across all injections in a particular sample set, is known as GeneScan™ 500 ROX™ Size Standard (Applied Biosystems, Warrington, UK) and is tagged with a red dye (9). The fluorescent tags are then excited by a laser and detected by a CCD camera during analysis. This fluorescent detection method has been shown to be reliable for forensic samples (8).

The sizing of alleles and the determination of the genotype are accomplished via comparison of allelic ladder base pairs to the base pair sizes measured for the sample alleles (10). Signal intensity, measured in relative fluorescent units (rfu), is also a relevant factor in determining genotypes and allele calls. In single donor profile, a

heterozygous locus is expected to have two peaks of equal intensity. While a homozygote locus is expected to have a single peak twice the intensity of the heterozygote peaks.

The development of the polymerase chain reaction has allowed a great deal of progress to be made in the field of forensic science (8). Degraded samples and limited samples, commonly encountered in forensic science, may not possess DNA in sufficient quantity or quality for testing by RFLP or protein electrophoresis. STR DNA analysis provides an excellent option for forensic scientists faced with poor quality samples. STR typing is a superior tool to aid in solving crimes today.

CHAPTER ONE

Evaluation of minor components in mixed biological samples typically found in forensic scenarios

Mixtures of biological fluids, such as blood, semen and saliva, are frequently the primary source of biological evidence in sexual assaults and other violent crimes (11). For example, sexual assaults in San Jose, CA, averaged 34.6% of all the violent crimes (homicide, robbery and rape) for June 2000 and 31% for June 1999 (San Jose Police Department Master Report, June 2000). Homicides and sexual assault cases frequently produce mixed biological evidence involving both the victim's epithelial cells and the suspect's semen. A female victim's epithelial cells may be in the form of saliva or vaginal secretions. In cases involving oral copulation, saliva from a male suspect may also be present. Blood from the suspect, the victim, or both may also be present in some cases.

Interpretation of data from mixed samples can be confusing and difficult (12). In many mixture cases, the biological components are often present in varying proportions. Minor components may be present in the shadow of the major donor's components. In other cases, two donors may be present in equal amounts. Finally, if both donors share one or more common alleles at a single locus, it may be difficult or impossible to determine if a minor donor's contribution is present in a sample. At a given locus, assuming a single source, one would expect a single peak for a homozygote and two equal intensity peaks from a heterozygote to show up in an electropherogram (13). If more than one individual contributed DNA to the sample in question, the analyst may

detect multiple allele peaks at a particular locus. Multiple allele peaks at a single locus are a clear indication that a mixture is present in the sample (14). As the number of alleles at a given locus increases, the ability to exclude a potential suspect decreases. In general, comparing the peak heights of alleles will help the analyst determine whether or not a true mixture is present. Peak height ratios that are less than 70% indicate that a mixed sample may be present. Single source samples rarely generate a peak height ratio of less than 70%. However, factors such as degraded samples, low input DNA, or primer binding site mutations may occasionally cause a peak height ratio to dip below 70% for single source samples.

In sexual assault cases involving semen, it is important to be able to separate sperm cells from other biological fluids in order to include or exclude the suspect from the evidence. Based on the DNA typing results, a suspect may be included or excluded from a particular sample. If the suspect is included, the DNA profile from the evidence contains alleles common to those in the suspect's profile. If he is excluded, the DNA profile for the semen evidence does not include alleles common to the suspect's profile and he is not considered a contributor to that evidentiary DNA profile. In some cases, more than one semen donor may be involved, and separating the semen component from the epithelial cell component may help to determine the number of assailants. Some commonly encountered biological mixtures, associated with violent crimes, include blood and semen, saliva and semen, and two semen donors. While semen can be separated from epithelial cells, mixed samples involving only saliva, vaginal secretions, skin cells,

and blood cannot be separated. Mixtures involving two or more semen donors cannot be separated either.

In addition to true mixed samples, there are several other aspects of the DNA analysis that may complicate interpretation (14). Samples which contain multiple allele peaks at a single locus must be carefully evaluated in order to prevent interpretation errors from peaks that may mimic true alleles such as "stutter" peaks, electronic spikes and pull-up peaks. "Stutter" peaks, caused by slipped strand mispairing, may appear in the n-4 base pair position, when n is the true allele peak (7). Minor alleles detected in the n-4 base pair position must be carefully considered in mixed samples. True "stutter" peaks have peak height ratios that are generally less that 15% of the main allele peak (13,14). Electronic spikes from the instrument power source can also produce peaks that may be called as alleles by the computer software. Electronic spikes generally appear in all four fluorescent dye colors and, with careful analysis, the analyst can identify the electronic spikes as such. Pull-up peaks also occur in the diagnostic range of the electropherograms. These peaks are the result of an outdated matrix, which normally adjusts for the fluorescent dye spectral overlap (14,15). An inexperienced analyst may mistake both pull-up peaks and electronic spikes for minor components in a mixture.

One can dramatically increase the discrimination power by choosing multilocus STR DNA analysis over a single locus PCR method. STR DNA analysis is currently the best technology available to most crime laboratories (3). It provides forensic scientists with the tools needed to differentiate between individuals. While other DNA analysis systems such as D1S80 and DQα may be less expensive, the discrimination power associated

with them is relatively low. STR analysis using the AmpF*l*STR Profiler Plus™ kit (Perkin-Elmer, Foster City, CA) allows for the amplification of nine STR loci and a sex determination locus, Amelogenin, in a single multiplex PCR reaction. The time it takes to amplify these ten loci is approximately the same amount of time it takes to amplify a single locus such as D1S80 or DQα. In addition, the instrument used to run the amplified PCR product, the ABI Prism ™ 310 Genetic Analyzer, allows for one sample to be analyzed in approximately 30 minutes, resulting in a high sample throughput.

While some work has been done to determine the reliability of the system, extensive studies on these loci and mixed samples have not been performed. The limits of detection for this kit have not been studied extensively before now (9). This research examines the concentration at which the minor component in a mixture ceases to present a profile across the nine loci evaluated. Correlations between the detection limits of the minor component and the different biological fluids have been examined.

Methods and Materials

Sample Preparation

To examine the detectability of minor components in mixed biological samples, mixtures of saliva, blood and semen at various volume:volume ratios were prepared. The Human Subjects-Institutional Review Board at San Jose State University granted approval for the use of human subjects in this research. Undiluted semen samples were obtained from twelve males. In most cases, samples from a single individual were pooled over several days in a sterile container. Three undiluted saliva samples and three blood samples were obtained from a total of six individuals. The liquid biological fluids were mixed in known ratios and spotted onto sterile 100% cotton cloth. The mixtures were prepared in triplicate using different individuals for each set of samples. The following sample mixtures were prepared in ratios of 25:1, 15:1, 10:1, 1:1, 1:10, 1:15, and 1:25; semen with semen, saliva with semen, and blood with semen. With three different types of mixtures, seven ratios, and three sets for each mixture type, a total of 63 samples were prepared. The samples were dried overnight at room temperature and then frozen at -18°C until the samples could be extracted.

Due to the different chemical composition and viscosity of the biological fluids used in the mixture portion of this experiment, certain considerations were made. In order to obtain a consistent sample, the biological fluids were first mixed in a test tube or microcentrifuge tube. Due to the different viscosities of the three liquids, some apparent separation of the mixed liquids was observed once spotted on the cloth. In order to

account for this apparent separation, cuttings were taken in the shape of a triangle, similar to a slice of a pie. This triangular cutting was made in an attempt to sample all regions of the stain in a single cutting. The total area of the cutting was approximately $(5mm)^2$-$(8mm)^2$ and covered the entire spectrum of the spotted stain.

DNA Extraction Procedure

In order to obtain the DNA, it was necessary to extract it from the prepared stains. Semen/semen mixture sample cuttings were organically extracted after digestion in 500μL Digest Buffer (see Appendix), 15μL Proteinase K (10mg/mL)(Gibco BRL® Life Technologies, Gaithersburg, MD) and 20μL 1M Dithiothreitol (International Biotechnologies, Inc, subsidiary of Eastman Kodak Co., New Haven, CT) and incubated at 56°C for a minimum of four hours.

Blood/semen and saliva/semen mixture samples required a differential extraction prior to the organic extraction. Blood/semen and saliva/semen cuttings were soaked in 1mL sterile phosphate buffered saline (see Appendix) at 4°C for a minimum of two hours. The soak liquid was discarded and the cutting centrifuged at 9000 rpm for two minutes in a "piggyback" filter basket to create a pellet in the microfuge tube. The supernatant was removed and discarded, leaving 50μL in the microfuge tube. To this pellet, 500μL Digest Buffer (see Appendix) and 15μL Proteinase K (10mg/mL)(Gibco BRL® Life Technologies, Gaithersburg, MD) was added, and the samples incubated for one hour at 56°C. After centrifugation for five minutes, the supernatant was removed to a new sterile microfuge tube. This supernatant contains the epithelial cell, or non-sperm cell, fraction

of the sample. The remaining sperm pellet was washed five times with 1mL phosphate buffered saline and one time with 1mL sterile water. Once the final wash was complete and the supernatant removed, the sperm pellet was digested with 500μL Digest Buffer (see Appendix), 15μL Proteinase K (10mg/mL)(Gibco BRL® Life Technologies, Gaithersburg, MD) and 20μL 1M Dithiothreitol (International Biotechnologies, Inc, subsidiary of Eastman Kodak Co., New Haven, CT), and incubated for a minimum of two hours at 56°C.

All sample cuttings were subjected to organic extraction using a buffered chloroform/phenol solution (Shelton Scientific, Inc. Shelton, CT) followed by centrifugation at 9000 rpm for five minutes. The aqueous layer was placed in a Centricon®-100 (Amicon® bioseparations, Millipore Corporation, Bedford, MA) filled with 1.5mL TE buffer (see Appendix) and centrifuged at 2800 rpm for approximately 15 minutes. Two additional TE buffer wash steps, using 2mL each, followed the initial wash step. The Centricon®-100 retenate cup was inverted and centrifuged at 1800 rpm for two minutes. The volume of recovered DNA was then estimated and recorded.

DNA Quantitation

The concentration of the DNA was determined prior to the amplification process. Once extracted, the DNA samples were quantitated using one of the following methods: yield gel or QuantiBlot™ Human DNA Quantitation kit (Perkin-Elmer, Foster City, CA). Both protocols are briefly described here.

DNA yield gels were prepared using 0.4 g SeaKem LE agarose (FMC® BioProducts, Rockland, ME) and 40mL 0.5X TBE buffer (see Appendix). Samples and DNA quantitation standards were electrophoresed in a gel box at 60 volts for approximately one hour. Gels were then placed on a UV light box and photographed for interpretation. In order to estimate the concentration of DNA, the intensity of the unknown sample bands was compared to that of the standard bands.

The procedure for the QuantiBlot™ quantitation was performed based on the manufacturer's recommended protocols found in the product insert. Briefly, the QuantiBlot™ procedure is described below: QuantiBlot™ standards and each sample were prepared by mixing the sample with 150μL spotting solution then pipetting the solution into the vacuum apparatus (Life Technologies™) in which a Biodyne® B membrane (Gibco BRL® LifeTechnologies™ Inc, Gaithersburg, MD) was placed. The immobilization, hybridization, wash steps, and color development were followed according to the manufacturer's recommendations (QuantiBlot™ Kit product insert). The membrane was photographed and the DNA yield estimated by comparing color intensity of DNA quantitation standards to the unknown samples.

DNA Amplification

DNA amplification was achieved via the polymerase chain reaction (PCR), which exponentially copies regions of interest on the chromosomes prior to analysis. The AmpF*l*STR® Profiler Plus™ kit (Perkin-Elmer, Foster City, CA) is a multiplex kit that allows amplification of nine STR loci and Amelogenin in a single PCR reaction. A

master mix containing components found in the AmpF*l*STR® Profiler Plus™ kit was prepared in accordance with the manufacturers recommendations (AmpF*l*STR Profiler Plus™ User's Manual): 21μL Reaction Mix + 11μL Primers + 1μL AmpliTaq Gold™ (Perkin-Elmer) for each sample to be amplified. 30μL master mix was then aliquoted into GeneAmp® (Perkin-Elmer Applied Biosystems, Foster City, CA) 0.5mL sterile tubes and topped with one drop of mineral oil.

To each tube containing PCR master mix, 20μL of sample DNA/TE buffer was added with a target DNA concentration of between 0.8-1.5ng DNA in 20μL. The GeneAmp® tubes were placed into a PE 480 Perkin Elmer DNA Thermal Cycler (Perkin-Elmer) and amplified using the following program: initial incubation at 95°C for 11 minutes, 28 cycles (1 cycle= denature at 94°C for 1 minute, anneal at 59°C for 1 minute, extend at 72°C for 1 minute), extension at 60°C for 45 minutes and hold at 25°C. To ensure that the amplification reaction was working, a positive amplification control consisting of a known DNA sample (AmpF*l*STR™ control DNA 9947A) and a negative control consisting of TE buffer were run with each set of amplifications.

Capillary Electrophoresis of DNA

In order to examine the detection level of minor components in mixed samples, all nine loci present in the AmpF*l*STR Profiler Plus™ kit were analyzed using the ABI Prism™ 310 Genetic Analyzer (Perkin-Elmer, Foster City CA) capillary electrophoresis instrument. 1.5μL of amplified DNA product was added to 25μL deionized formamide

(Amresco, Solon, OH). The samples were then placed into the PE 480 Thermal Cycler (Perkin-Elmer) for a minimum of three minutes at 95°C to denature the double stranded DNA. The samples were removed and snap cooled in an ice water bath for a minimum of three minutes to prevent re-annealing. A tube containing the AmpF*l*STR Profiler Plus™ allelic ladder was also prepared in the same manner and tested with the mixed samples. The samples and allelic ladders were compared by the GeneScan® (Perkin-Elmer, Foster City, CA) and GenoTyper® (Perkin-Elmer, Foster City, CA) software programs and genotypes were assigned to the samples (10,12).

Analysis and interpretation of DNA analysis

In developing analysis parameters for this research, the following guidelines were used to interpret the data for the mixture portion of this project. Minimum analysis parameters of 50 rfu (relative fluorescent units) and five second sample injection time were used for this portion of the data analysis. A ten second injection time was permitted if the sample concentration was too weak using a five second injection. Samples with ten second injection times are noted in the results.

Typing results were scored from the electropherograms indicating major and minor alleles at each locus evaluated. These results were summarized on tabular sheets (data not shown). For cases in which both donors shared a common allele at a locus, the peak height ratio was calculated in order to determine whether or not the minor donor was contributing to the common allele.

$$\frac{\text{peak height of smaller allele}}{\text{peak height of larger allele}} \times 100 = \text{peak height ratio}$$

If a ratio of less than 70% was calculated, and the largest allele was the one both donors had in common, the minor donor was considered a contributor. If the peak height ratio was greater than 70%, then the minor donor was excluded as being a contributor to that allele for the purposes of this research.

Reference samples from each donor used in this study were typed using the AmpF*l*STR Profiler Plus™ kit. Mixture sample results were evaluated on the basis of whether or not the sample presented the profile consistent with the major contributor of the mixture. For example, in a saliva/semen mixture in a ratio of 10:1, the mixture typing results were compared to the results from the saliva donor's reference sample. The number of loci that showed evidence of the minor contributor, the semen donor, were tabulated (data not shown). So, if the semen donor's alleles were detected at two loci (D3S1358 and D18S51 for example), the score for that particular sample was two out of a possible nine. Amelogenin was not considered in these calculations.

Once scores for each individual in a particular ratio and mixture type (i.e. 10:1, saliva/semen) were tabulated, the mean and standard deviation for each category were calculated. The 95% confidence interval was also calculated for each category.

This process was duplicated for each ratio and mixture type. Mixed samples that were separated into epithelial cell and sperm cell fractions were evaluated separately. Each fraction was evaluated for both the male and the female donors.

In addition, the limit of detection as defined by Wallin et al. (9) was determined for each mixture type. The detection limit is defined as the last sample, at either end of the

mixture ratio, in which the profile appears to have more than one source. Epithelial cell fractions and sperm cell fractions were evaluated separately.

Results

A total of 63 mixed samples were typed using the AmpF*l*STR Profiler Plus™ kit. The first mixture type to be evaluated was a mixture of two semen donors. A total of six individuals (n=6) were used to prepare mixture ratios including 25:1, 15:1, 10:1, and 1:1, 1:10, 1:15, and 1:25. The number of loci in which the minor donor's type was detected was calculated for each of the ratios and the mean computed. A 95% confidence interval was also calculated for each mean. The results for the semen/semen mixture ratios may be found in Figure 1.

The second mixture type to be evaluated was a mixture of saliva and semen donors. A total of six individuals, were used to prepare three sets (each set consisting of a female and a male) of mixture ratios including 25:1, 15:1, 10:1, 1:1, 1:10, 1:15 and 1:25. The number of loci in which the minor donor's type was detected was calculated for each ratio in the epithelial cell fraction and the mean computed. The same calculations were performed for the sperm cell fraction. The 95% confidence interval was also calculated for each mean. The results for the saliva/semen mixture ratios for the epithelial cell fraction may be found in Figures 2a and 2b. Results for the sperm cell fraction are presented in Figures 3a and 3b.

The final mixture type to be evaluated was a mixture of blood and semen donors. A total of six individuals, were used to prepare three sets (each set consisting of a female and a male) of mixture ratios including 25:1, 15:1, 10:1, 1:1, 1:10, 1:15 and 1:25. The number of loci in which the minor donor's type was detected was calculated for each

ratio in the epithelial cell fraction and the mean computed. The same calculations were performed for the sperm cell fraction. The 95% confidence interval was also calculated for each mean. The results for the blood/semen mixture ratios for the epithelial cell fraction may be found in Figures 4a and 4b. Results for the sperm cell fraction are presented in Figures 5a and 5b.

One sample did not yield any results even upon re-extraction. Two samples were injected for ten seconds in order to produce acceptable results. These samples are scored along with the five second injections but are noted here as being ten second injections.

The limits of detection were calculated for each of the mixture types per the definition described by Wallin et al. (9). Individual limits were determined for samples with epithelial cell fractions and sperm cell fractions. The results are presented in Tables 2a, 2b, and 2c.

A distinct difference in the amount of DNA, by volume, was present in each biological fluid used in this study. A reference sample consisting of 25μL of the biological fluid from each person involved in the study was extracted and quantified. The quantitation results for each body fluid were averaged and the results are presented in Table 3.

Conclusions and Discussion

In general, the number of minor donor loci detected in the semen/semen mixture profiles increased as the ratio of major to minor donor contribution approached 1:1. This is not surprising given that the mixtures are comprised of only semen samples, and therefore there is no large discrepancy in the DNA concentrations of the two mixture components.

Differential extraction is a chemical method of separating the sperm cell fraction of a mixed sample from the non-sperm or epithelial cell fraction. Once the differential extraction is performed, one expects to detect only the epithelial cell donor's type in the epithelial cell fraction. Only the semen donor's type is expected in the sperm fraction. If multiple donors are present in any given fraction, some or all of the donors may be detected in that fraction. If a mixed sample has a very high concentration of one biological fluid, that fluid donor's type may "carry over" into the other fraction. For example, if there is a very high concentration of semen in a sample, one may detect the semen donor's genetic profile as a minor type in the epithelial cell fraction of the sample.

In both the blood/semen and saliva/semen mixtures, very few minor donor loci were detected in the sperm fraction when the minor donor was female. The sperm fractions from the blood/semen and saliva/semen mixtures were evaluated based on the number of loci that exhibited evidence of the minor donor's genetic type. In covering the range of dilutions, the sperm fractions were evaluated in cases where the female was the minor donor and in other instances when the male was the minor donor. In cases when the

female was the minor component in the sperm fraction, her profile was rarely detected. This result is not unexpected given that this is the sperm fraction and the difference in DNA concentration ranges between the major semen component and the minor saliva and blood components. In cases when the minor donor was the male, however, the average number of loci that were detected in the mixture profiles was 7.87. This indicates that when the female was the major donor and the male the minor donor, her profile was detected with the minor male donor's profile in approximately eight of nine loci in most cases.

In epithelial cell fractions when the male was the minor donor, the general trend again was that as the ratio approached 1:1, the number of minor loci being detected in the profile increased. The epithelial cell fraction from the blood/semen and saliva/semen mixtures were also evaluated based on the same criteria listed above. In epithelial cell fractions covering the range of dilutions, the fractions were evaluated in cases when the female was the minor contributor and cases when the male was the minor contributor. In cases when the male made up the minor component in the epithelial cell fraction, his genetic profile became more prevalent as the mixture ratio approached 1:1. This is not surprising given that semen samples have such a high concentration of DNA compared to blood or saliva, even though one would expect the female type to be predominant given that this is an epithelial cell fraction

In cases involving the female as the minor donor to the epithelial cell fraction, the overall trend appears to be an increase in minor loci detected as the ratio approaches 1:1. While there is a slight decrease in the mean number of loci detected at the 10:1 ratio in

the semen/saliva mixture, the overall trend is that the number of minor loci detected increases. This trend is seen clearly in the semen/blood mixture results. This result is not unexpected since the amount of DNA in semen is likely to overpower the female type in most cases. And, since this is an epithelial cell fraction, one would expect the female's profile to be detected as well. The semen, blood and saliva donors in this study are not necessarily a representative sampling of the population and thus, additional studies using a larger sample population should be performed.

A 95% confidence interval was computed for the ratios in each category of mixtures. This is depicted on the graphs as the upper and lower points on either end of the mean. There is a 95% probability that the true mean will fall within the integers that make up the high and low ends of the 95% confidence interval.

The limits of detection as defined by Wallin et al. (9) indicate that, in most cases, the minor component may be detected in a mixed sample, even when the major component is present in concentrations 15-25 times higher than the minor component. In considering the upper and lower limits of detection for 30 samples, 60 data points were evaluated. Only six points indicated a detection limit less than 1:15/15:1. These seemingly anomalous results may be due to sampling errors or an incomplete differential extraction. Twelve samples exhibited no evidence of a mixture in the sperm fraction of the mixture, which is not unexpected due to the differential extraction process. In each of the twelve cases, the genetic profile matched that of the semen donor. One sample, a saliva/semen mixture 25:1, did not yield any results and was excluded from these calculations.

The calculations for the limits of detection evaluate only the high and low ends of the mixture ratio spectrum, and do not account for any of the ratios that fall in between these high and low values. For example, a set of samples with limits of detection at 25:1 and 1:25 may not exhibit evidence of a mixture in the 10:1 ratio. The limits of detection are informative on a qualitative basis rather than being quantitative as in the calculations performed in this study.

Semen has approximately 68 times as much DNA as does liquid blood and more than 100 times as much DNA as liquid saliva. Based on the extraction and quantitation of twelve semen samples, three blood samples, and three saliva samples from individuals involved in this study, a significant discrepancy in the amount of DNA present in each biological fluid was observed. Liquid saliva has less DNA by volume than liquid blood. These findings will affect the results of the mixtures since the ratios were prepared by volume alone. One would expect the profile from the semen donor to be detected even when it is very dilute, due to the high concentration of DNA present in semen (18). The concentration differences between blood and saliva are much less pronounced, and therefore these liquids may not be detected in dilute samples.

These findings are particularly relevant to the case analyst working a complicated mixture case. For example, imagine a case in which the only evidence is a sexual assault evidence collection kit containing vaginal swabs from a victim who showered after her attack and waited twelve hours before reporting the assault. In a case like this, the fact that the suspect's semen may be present in only small amounts should not discourage the forensic scientist. Based on this study, one may still find the minor semen donor's DNA

profile on the vaginal swab, due simply to the fact that the concentration of DNA in the semen is very high compared to the victim's epithelial cell DNA. In addition, based on these studies, the scientist has a reasonable chance at obtaining results that will implicate or vindicate the suspect, due to the sensitivity of the AmpF*l*STR Profiler Plus™ typing system.

CHAPTER TWO

Degradation of target DNA in blood and semen samples after exposure to ultraviolet light, sunlight, and fluorescent light

Forensic science evidence is often comprised of biological fluids, such as blood and semen, collected from objects or persons involved in a crime. Bodily fluids from the suspect, the victim, or both may be present. Deoxyribonucleic acid (DNA) from blood and semen can provide the genetic profile of the individual from whom the sample originated, yielding an invaluable tool to the investigators on the case.

There are several potential factors found at crime scenes which may affect the quality of biological evidence; light is one such factor. Therefore, any crime that involves blood or semen evidence may be impacted if the sample is subjected to conditions that prevent the analyst from obtaining a complete genetic profile. There are essentially three types of light that may be found in a crime scene situation: fluorescent light, natural sunlight and ultraviolet light. One or all of these types of light may have some degradative effect on biological samples such as blood and semen, potentially affecting the laboratory analyses. The light sources examined in this study include ultraviolet (UV) light from a biosafety hood, sunlight, and fluorescent light. These three types of light were chosen as a representative sample of possible environments from which crime scene samples may be collected. For example, the fluorescent light conditions mimic a crime scene in a home or business. Sunlight conditions mimic an outdoor crime scene. And finally, the UV light from the biosafety hood was included because it is widely thought that UV light

breaks down DNA to negligible amounts and is used in sterilization procedures (19, 20). Length of exposure is also a factor since forensic investigations may take several months to complete. This is especially important to evaluate since the forensic evidence may not always be collected immediately after the incident.

Short tandem repeat (STR) analysis is a robust technique that may be used when the condition of the DNA sample is less than ideal. Conventional methods such as restriction fragment length polymorphism (RFLP) and protein electrophoresis are outdated and are not well suited for biological samples that are very old or degraded (4,16). Protein electrophoresis techniques rely on the integrity of the protein and RFLP analysis requires high molecular weight DNA in high concentrations (17). As a sample gets older, is stored under humid, warm conditions or is exposed to ultraviolet rays, the protein may break down, and DNA may be broken into small pieces. STRs have been shown to reliably type degraded and aged samples (5, 9,16) and samples that are present in minute quantities without the limitations of protein electrophoresis or RFLP (7,16). STRs have been used to identify human remains from mass disasters (5,16) and biological evidence from crime scenes.

The high rate of polymorphism, or variability among individuals, makes STRs an excellent choice for human identification (4). While innovations in polymerase chain reaction (PCR) have led to improved DNA analysis techniques such as DQα and D1S80, these loci alone may not be discriminating enough in some criminal cases. The AmpF*l*STR Profiler Plus™ kit (Perkin-Elmer, Foster City, CA) incorporates the following nine STR loci and Amelogenin in a single multiplex PCR reaction: D3S1358,

vWA, FGA, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820. This combination of carefully chosen loci allows for enormous discrimination power with respect to forensic science cases involving degraded samples (5,16).

In cases involving either degraded DNA that is broken into small pieces or intact DNA present in very small amounts, a condition known as stochastic effects may be observed (14). That is, due to a very low concentration of DNA, one allele may be preferentially amplified during PCR over the second allele at the same locus (1,9). Stochastic effects are only observed in heterozygotes. The result of this preferential amplification is that one allele may be detected and typed, while the second allele is not. The genotype at the affected locus then appears as a homozygote when, in fact, it is truly a heterozygote (5,9).

Very little research has been published, with respect to the AmpF/STR Profiler Plus™ loci, which specifically evaluates the effects of types of light on biological fluids such as blood and semen. Validation of the kit has been performed, and some data has been presented to demonstrate that the kit reliably types degraded samples (9). While extensive degradation studies have been performed on other PCR DNA typing systems (18), very few studies on these STR loci in particular have been completed. This research will determine if a pattern of degradation, demonstrated by the inability to amplify certain STR loci, occurs after blood and semen samples are exposed to different light sources over a range of time intervals. Therefore, the results of this research will fill a research void in forensic science with respect to this type of DNA STR testing.

Methods and Materials

Light Sources

The combination of the different light sources and time interval were examined in order to evaluate whether or not the light sources and/or the exposure intervals had complete degradative effect on DNA in either the blood or semen samples. The first light source considered was the UV light from a 30-Watt Germicidal bulb (19, 20)(model G30T8, Sankyo Denki, Japan) in a LabConco Purifier™ Class II Safety Cabinet biosafety hood located in the Santa Clara County Crime Laboratory. The samples were placed 53 cm from the bulb. This lighting source emits ultraviolet rays at 253.7nm (ultraviolet output of approximately 19 Watts (two bulbs at 9.5 Watts each), average useful life of approximately 6500 hours, lamp current of 0.355 Amps). It should be noted that this G30T8 bulb has a reduced ultraviolet output of approximately 70% of the initial output at 5000 hours. Based on a useful lifetime of 6500 hours (270 days/24 hours per day) it is likely that the samples in the biosafety hood were exposed to less than 70% of the initial ultraviolet rays for the majority of the 13 month interval (Bulbman, Reno NV). The actual output of the germicidal bulbs was measured approximately 1.5 years after the last sample was collected. The measurements were taken using UVA detector model PMA2111 and UVB detector model PMA2102 (Solar Light Co. Inc, Philadelphia, PA). The actual UV output of a single bulb was found to be 0.012 mW/cm^2 for UVA and 2.49 $\mu W/cm^2$ for UVB. At 70% efficiency, the predicted output of the two bulbs in the hood is $0.87mW/cm^2$.

The second light source was chosen in an attempt to mimic the effects of natural sunlight, without subjecting the samples to other weather conditions such as precipitation, wind or temperature (18). This was achieved by placing the set of ten samples on a table directly underneath a lead glass window inside a home in Santa Cruz, CA. This window allowed approximately 86% of the visible light, 65% of the UVA and 6% of the UVB rays from the sun to pass through the window (data not shown). The light passing through this window was determined by the UV detectors mentioned above.

The third light source considered was that of fluorescent lights. In this case the samples were placed on a lab bench in the Santa Clara County Crime Lab at room temperature (18). The samples were positioned 53cm inches from the 32-Watt bulb (mean output of 2650 lumens, rated average lifetime of 20,000 hours, General Electric Trimline T8, F32T8-SP35). The light emitted from these bulbs was determined to be 17.2μE/m^2s for visible light, 0.006 mW/cm^2 for UVA and 0.18 MED/hr for UVB.

Sample Preparation

In order to examine the degradative effects of three different light sources on DNA in biological fluids, numerous representative samples were prepared. The Human Subjects-Institutional Review Board at San Jose State University granted approval for the use of human subjects in this research. Five males donated undiluted semen samples. In most cases, an individual's sample was collected over several days by pooling together several contributions in a sterile container. Five different individuals, four females and one male, contributed approximately 7mL of a liquid blood sample.

A portion of each liquid sample, five blood and five semen, was spotted onto strips of sterile, white 100% cotton cloth in triplicate, resulting in a total of 30 strips. One complete set of ten samples, five blood and five semen, was then placed under the appropriate light source.

Sample Exposure

A sampling schedule was developed to evaluate the effect of time on the degradation of STR loci in the biological fluid stains. The samples were placed under their respective light sources on January 2, 1998. The samples were not removed from their exposure positions except during sample collection. Beginning two weeks after the initial exposure, a portion of each stain was removed from each sample under the three light conditions. Subsequent collection intervals included: 1 month, 2 months, 3 months, 6 months, 9 months, and 13 months. The samples were frozen at approximately -18° C until they could be extracted. It is assumed that this freezing procedure prevented further degradation of the sample. None of the samples were protected from dust or contaminants in the exposure locations.

DNA Extraction Procedure

An organic extraction procedure was used to obtain DNA from the light exposed specimens. Samples were digested in 500μL Digest Buffer (see Appendix), and 15μL Proteinase K (10mg/mL)(Gibco BRL® Life Technologies, Gaithersburg, MD). 20μL 1M Dithiothreitol (International Biotechnologies, Inc, subsidiary of Eastman Kodak Co.,

New Haven, CT) was added to semen samples prior to incubation at 56°C for a minimum of four hours.

Samples were then extracted using a buffered chloroform/phenol solution (Shelton Scientific, Inc. Shelton, CT) followed by centrifugation at 9000 rpm for five minutes. The aqueous layer was transferred to a Centricon®-100 (Amicon® bioseparations, Millipore Corporation, Bedford, MA), filled with 1.5mL TE buffer (see Appendix) and centrifuged at 2800 rpm for approximately 15 minutes. Two additional TE buffer wash steps, using 2mL each, followed the initial wash step. The Centricon®-100 retenate cup was inverted and centrifuged at 1800 rpm for two minutes. The volume of recovered DNA was then estimated and recorded.

DNA Quantitation

The concentration of the DNA was determined prior to the amplification process. Once extracted, the DNA samples were quantitated using one of the following methods: yield gel or QuantiBlot™ Human DNA Quantitation kit (Perkin-Elmer, Foster City, CA). Both protocols are briefly described here.

DNA yield gels were prepared using 0.4 g SeaKem LE agarose (FMC® BioProducts, Rockland, ME) and 40mL 0.5X TBE buffer (see Appendix). Samples and DNA quantitation standards were electrophoresed in a gel box at 60 volts for approximately one hour. Gels were then placed on a UV light box and photographed for interpretation. In order to estimate the concentration of DNA, the intensity of the unknown sample bands was compared to that of the standard bands.

The procedure for the QuantiBlot™ quantitation was performed based on the manufacturer's recommended protocols found in the product insert. Briefly, the QuantiBlot™ procedure is described below: QuantiBlot™ standards and each sample were prepared by mixing the sample with 150μL spotting solution then pipetting into the vacuum apparatus (Life Technologies™) in which a Biodyne® B membrane (Gibco BRL® LifeTechnologies™ Inc, Gaithersburg, MD) was placed. The immobilization, hybridization, wash steps, and color development were followed according to the manufacturer's recommendations (QuantiBlot™ Kit product insert). The membrane was photographed and the DNA yield estimated by comparing color intensity of DNA quantitation standards to the unknown samples.

DNA Amplification

DNA amplification was achieved via the polymerase chain reaction (PCR) that exponentially copies the regions of interest on the chromosomes prior to analysis. The AmpF*l*STR Profiler Plus™ kit (Perkin-Elmer, Foster City, CA) is a multiplex kit that allows amplification of nine STR loci and Amelogenin in a single PCR reaction. A master mix containing components found in the AmpF*l*STR Profiler Plus™ kit was prepared in accordance with the manufacturer's recommendations (AmpF*l*STR Profiler Plus User's Manual): 21μL Reaction Mix + 11μL Primers + 1μL AmpliTaq Gold™ (Perkin-Elmer) for each sample to be amplified. 30μL master mix was then aliquoted

into GeneAmp® (Perkin-Elmer Applied Biosystems, Foster City, CA) 0.5mL sterile tubes and topped with one drop of mineral oil.

To each tube containing this PCR master mix, 20μL of sample DNA/TE buffer was added with a target DNA concentration of between 0.8-1.5ng DNA in 20μL. The GeneAmp® tubes were placed into a PE 480 Perkin Elmer DNA Thermal Cycler (Perkin-Elmer) and amplified using the following program: initial incubation at 95°C for 11 minutes, 28 cycles (1 cycle= denature at 94°C for 1 minute, anneal at 59°C for 1 minute, extend at 72°C for 1 minute), extension at 60°C for 45 minutes and hold at 25°C. To ensure that the amplification reaction was working, a positive amplification control consisting of a known DNA sample (AmpF*l*STR™ control DNA 9947A) and a negative control consisting of TE buffer were run with each set of amplifications.

Capillary Electrophoresis of DNA

In order to examine the amount of degradation caused by various light sources over time, all nine loci present in the AmpF*l*STR Profiler Plus™ kit were analyzed using the ABI Prism ™ 310 Genetic Analyzer (Perkin-Elmer, Foster City CA) capillary electrophoresis instrument. 1.5μL of amplified DNA product was added to 25μL deionized formamide (Amresco, Solon, OH). The samples were then placed into the PE 480 Thermal Cycler (Perkin-Elmer) for a minimum of three minutes at 95°C to denature the double stranded DNA. The samples were removed and snap cooled in an ice water bath for at least three minutes to prevent reannealing. A tube containing the AmpF*l*STR

Profiler Plus™ allelic ladder was also prepared in the same manner and tested along with the degradation experimental samples. The samples and allelic ladders were compared by the GeneScan ® (Perkin-Elmer, Foster City, CA) and GenoTyper® (Perkin-Elmer, Foster City, CA) software and genotypes were assigned to the samples (10, 12).

Analysis and Interpretation of DNA Analysis

In developing analysis parameters for this research, the following guidelines were used to interpret the data for the degradation portion of this project. Minimum analysis parameters of 50 rfu (relative fluorescent units) and five second sample injection time were used for this portion of the data analysis. A ten second injection time was permitted if the sample concentration was too weak using a five second injection. Samples with ten second injection times are noted in the results.

In order to evaluate the amount of degradation present in the blood and semen samples, a similarity index was calculated.

$$\frac{\text{total loci typed}}{\text{total loci attempted}} \times 100 = \text{percent similar loci}$$

The number of loci that yielded results was compared to the total number of loci possible. The similarity index was first calculated for individual samples at each time interval, and then the individual results were averaged. Comparisons were then made between the results from the different light sources. Samples exposed to ultraviolet light from the biosafety hood were compared to samples exposed to sunlight. UV light samples were then compared to the fluorescent light samples, and the sunlight samples were compared

to the fluorescent light samples. Samples that only yielded results with ten second injection times are reported along with the five second injection samples. Samples that presented an incomplete profile are reported as such.

Results

Blood samples did not show degradation, or decreased ability to amplify STR loci, after long term exposure to ultraviolet light, sunlight, or fluorescent light. Twenty-one samples from five blood donors and five semen donors were amplified with the AmpF*l*STR Profiler Plus™ kit at full volume and typed on the ABI Prism 310 Genetic Analyzer. All 105 of the blood samples (35 under UV light, 35 under sunlight, and 35 under fluorescent light) yielded results across all nine STR loci and Amelogenin during intervals ranging from 2 weeks through 13 months exposure (Figure 6). None of the 105 blood samples required a 10 second injection time for suitable results to be obtained.

Semen samples did not show an appreciable amount of degradation at STR loci after long-term exposure to sunlight or fluorescent light. All 70 of the semen samples exposed to the sunlight and the fluorescent light yielded results at all nine loci and Amelogenin.

Evidence of degradation was observed with six of the semen samples exposed to ultraviolet light over long periods of time. Of the 35 UV semen samples, six samples failed to present a complete profile across all nine loci and Amelogenin. Five produced partial profiles and one did not yield any acceptable results. Three of these samples showed evidence of stochastic effects and preferential amplification. One of the six samples was exposed for six months, three were exposed for nine months, and the final two samples were exposed for 13 months (Figure 7). The samples that did not produce full profiles originated from a total of three individuals. Three of the six samples were from a single individual, one was from a second individual and the remaining two

samples were from a third individual. Eight semen samples were injected for ten seconds in order to obtain suitable results.

The sample that did not yield results under established conditions did yield partial results at six loci when 3μL PCR product was used in place of the standard 1.5μL amplified product (data not shown). This was not re-extracted because additional sample was not available.

Conclusions and Discussion

Degradative effects, with respect to the ability to amplify STR loci, were not observed on blood samples exposed to the three light sources for exposure intervals up to 13 months. In addition, sunlight and fluorescent light had no degradative effect on semen samples exposed to these light sources over 13 months. Based on these findings, one may conclude that reliable STR DNA typing results may be obtained from blood and semen samples exposed to these light conditions, even after significant periods of time.

Ultraviolet light does appear to have some degradative effects on semen samples up to and after a period of six months. No degradative effect was observed on semen samples at three months exposure time and only one of five samples exhibited degradative effects at the six month interval.

As the DNA is broken into smaller pieces by the UV radiation, the largest loci will be affected first (5); smaller loci will be affected only as the severity of degradation increases (9). The ultraviolet light source, while not emitting UV rays at the highest possible output, appears to have a degradative effect on the semen samples. The loci most affected by the UV exposure were generally the largest of the nine loci. D18S51 (273-341bp) and D7S820 (258-294bp) were affected in all six cases, FGA (219-267bp) in four, and D13S317 (206-234bp) in three. D21S11 (189-243bp), vWA (157-197bp), D5S818 (135-171bp), and D8S1179 (128-168bp) were each affected in two cases. The smallest locus, D3S1358 (114-142bp) was affected in only one case. Higher rates of degradation were expected due to the long exposure times and the samples' proximity to

the light source. The cause of the relatively low rates of degradation may be the decreased UV output of the germicidal lamps due to the fact that the bulbs were likely in place much longer than the recommended 6500 hours.

Three semen samples exhibited evidence of stochastic effects and preferential amplification (18). The genetic profile of each individual was determined in this research so the number of loci affected by stochastic effects was easily verified. While small STR product sizes reduce the chance of preferential amplification, evidence of this effect is present in six of the semen samples that presented incomplete profiles (9). All three samples exhibiting stochastic effects originated from a single individual with exposure intervals of six months, nine months and thirteen months. Of the 27 STR loci, excluding Amelogenin, possible in three samples with nine loci for each sample, seven loci yielded incomplete results. Of these seven loci, four exhibited evidence of stochastic effects. Table 4 summarizes the results from the samples that exhibited stochastic effects. Note that the loci affected, D18S51, D7S820 and FGA, are the largest loci in the multiplex and therefore are expected to be affected by the UV radiation first.

In summary, semen and blood appear to be hardier than expected under sunlight and fluorescent light conditions. Ultraviolet light, sunlight, and fluorescent light had no observable degradative effect on blood samples exposed from two weeks through 13 months. Sunlight and fluorescent light had no degradative effect on semen samples during the same time intervals. Some degradation was expected with the sunlight samples due to the UVA and UVB rays emitted from the sun (18) even with the reduced rate of exposure from the glass filters. Minimal degradation was expected with the

fluorescent lights due to the time frame of exposure and the temperature under which they were stored. Since the fluorescent lights produce a negligible amount of UV rays, the expected degradation would likely be due to temperature rather than light.

While DNA typing results may also be obtained from semen samples under the same conditions, one should not expect a full genetic profile to be present if the sample has been exposed to an ultraviolet light source for any length of time. Ultraviolet light appears to have some degradative effects on semen samples when exposure times are greater than or equal to six months. While it appears that the germicidal lamp used in this experiment was not operating at its highest possible capacity, degradative effects were observed in six of the thirty-five semen samples exposed to UV light. Three of these six samples exhibited evidence of stochastic effects and preferential amplification. The semen and blood donors in this study are not necessarily a representative sampling of the population and thus, additional studies using a larger sample population should be performed.

Table 1. Locus designation, chromosome location, common sequence motif, size range in base pairs, and dye label data for loci contained in the AmpF*l*STR Profiler Plus™ kit (excerpted from AmpF*l*STR Profiler Plus™ User's manual).

Locus Designation	Chromosome Location	Common Sequence Motif	Size Range (bp)[a]	Dye Label
D3S1358	3p	TCTA $(TCTG)_{1-3}$ $(TCTA)_n$	114–142	5-FAM
vWA	12p12-pter	TCTA $(TCTG)_{3-4}$ $(TCTA)_n$	157–197	5-FAM
FGA	4q28	$(TTTC)_3$ TTTT TTCT $(CTTT)_n$ CTCC $(TTCC)_2$	219–267	5-FAM
Amelogenin	X: p22.1–22.3 Y: p11.2	– –	107 113	JOE
D8S1179[b]	8	$(TCTR)_n$[c]	128–168	JOE
D21S11	21	$(TCTA)_n$ $(TCTG)_n$ [$(TCTA)_3$ TA $(TCTA)_3$ TCA $(TCTA)_2$ TCCA TA] $(TCTA)_n$	189–243	JOE
D18S51	18q21.3	$(AGAA)_n$	273–341	JOE
D5S818	5q21–31	$(AGAT)_n$	135–171	NED
D13S317	13q22–31	$(GATA)_n$	206–234	NED
D7S820	7q	$(GATA)_n$	258–294	NED

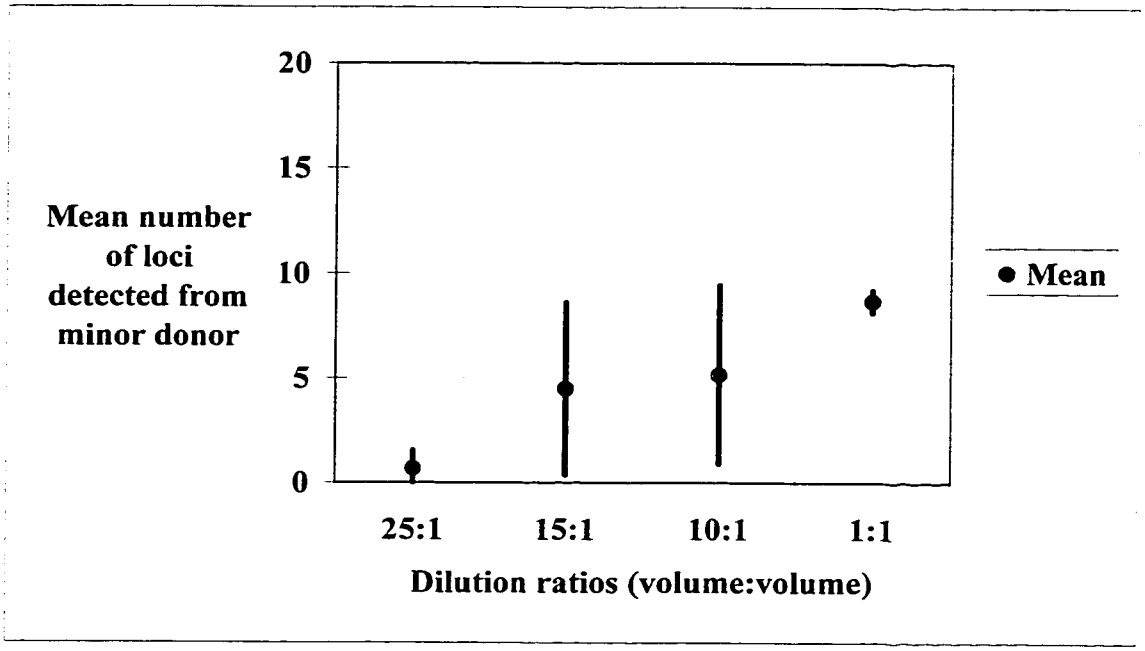

Figure 1. Semen/semen mixture evaluated for the number of loci detected from the minor semen donor. This graph shows the mean number of loci and the 95% confidence interval.

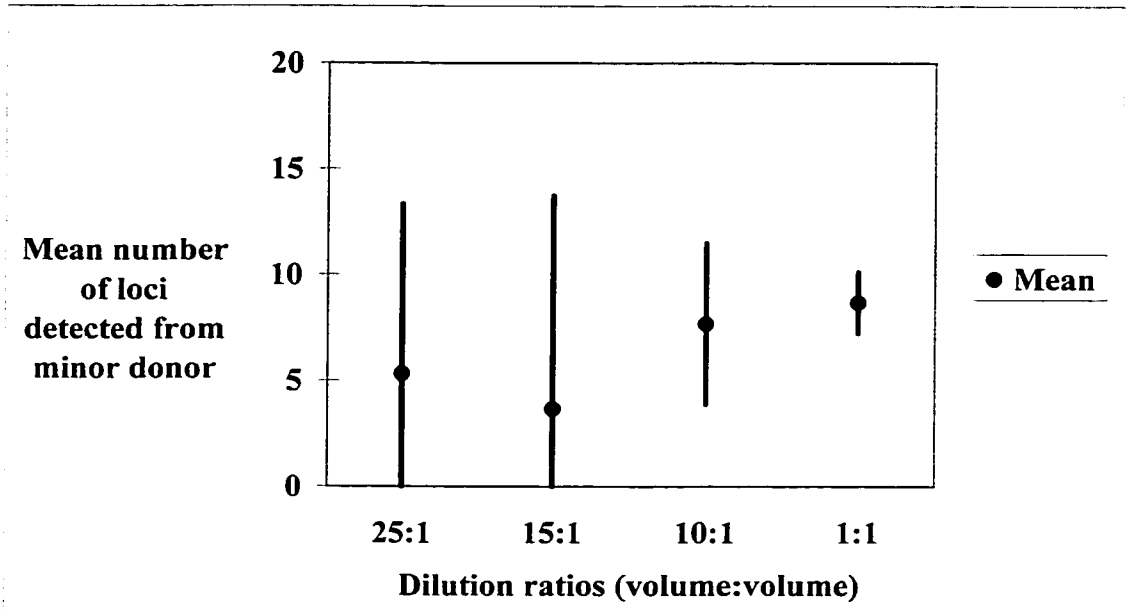

Figure 2a. Epithelial cell fraction of saliva/semen mixture when female (saliva) is major contributor and male (semen) is minor contributor. This graph shows the mean number of loci and the 95% confidence interval.

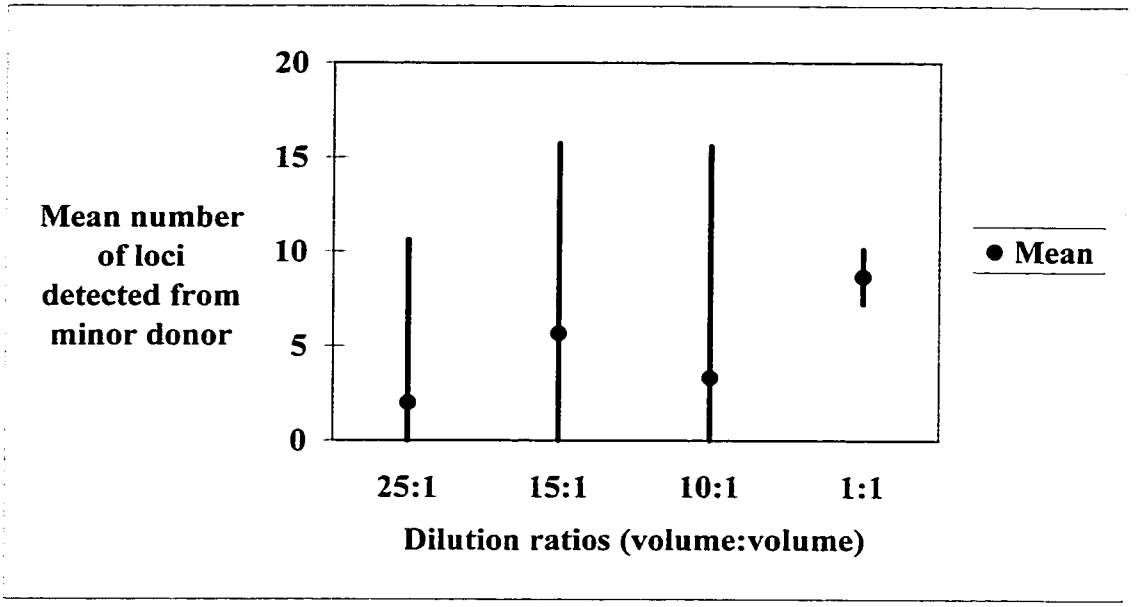

Figure 2b. Epithelial cell fraction of semen/saliva mixture when male (semen) is major contributor and female (saliva) is minor contributor. This graph shows the mean number of loci and the 95% confidence interval.

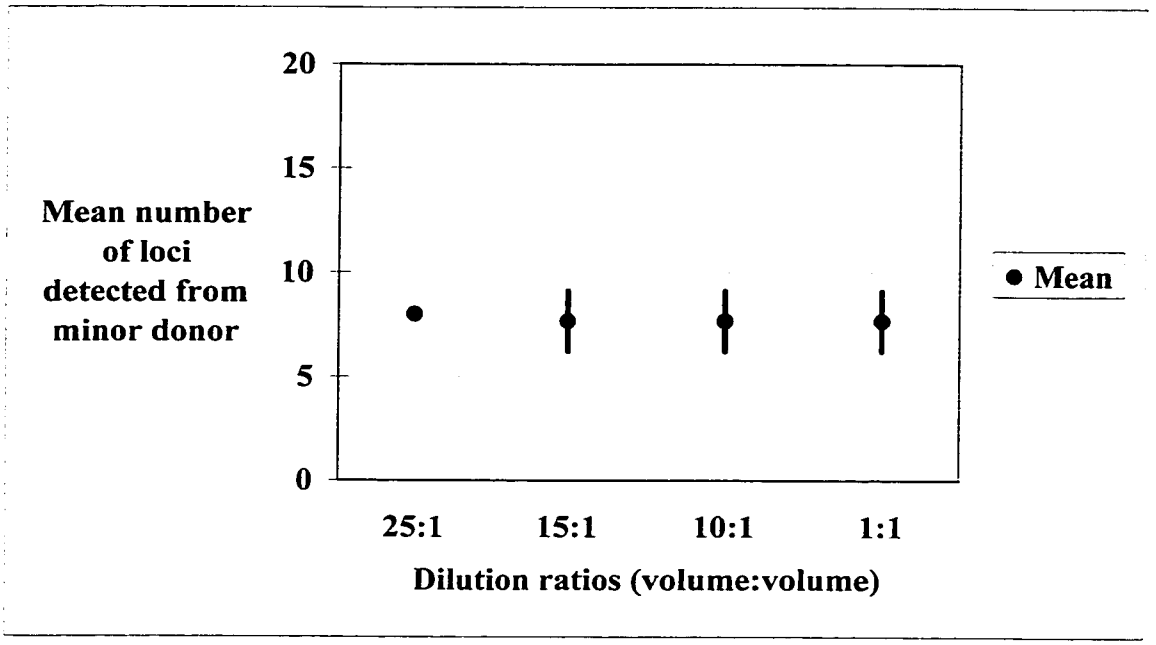

Figure 3a. Sperm fraction of saliva/semen mixture when female (saliva) is major contributor and male (semen) is minor contributor. This graphs shows the mean number of loci and the 95% confidence interval.
Note that the 25:1 ratio is the category in which one sample yielded no results and therefore n=2 for this ratio.

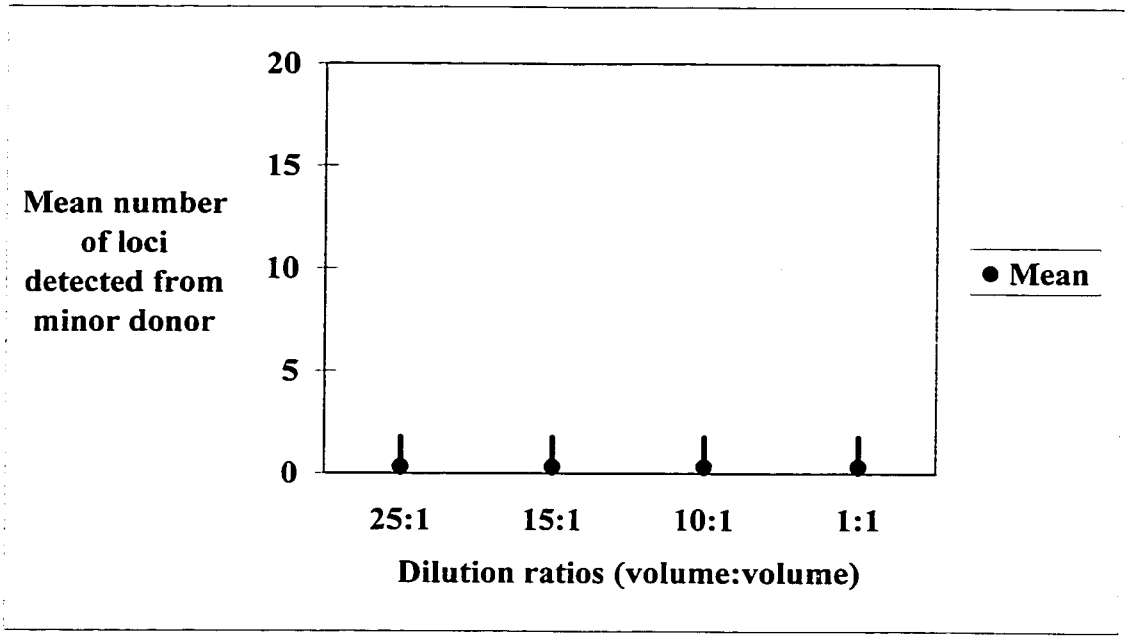

Figure 3b. Sperm fraction of semen/saliva mixture when male (semen) is major contributor and female (saliva) is minor contributor. This graphs show the mean number of loci and the 95% confidence interval.

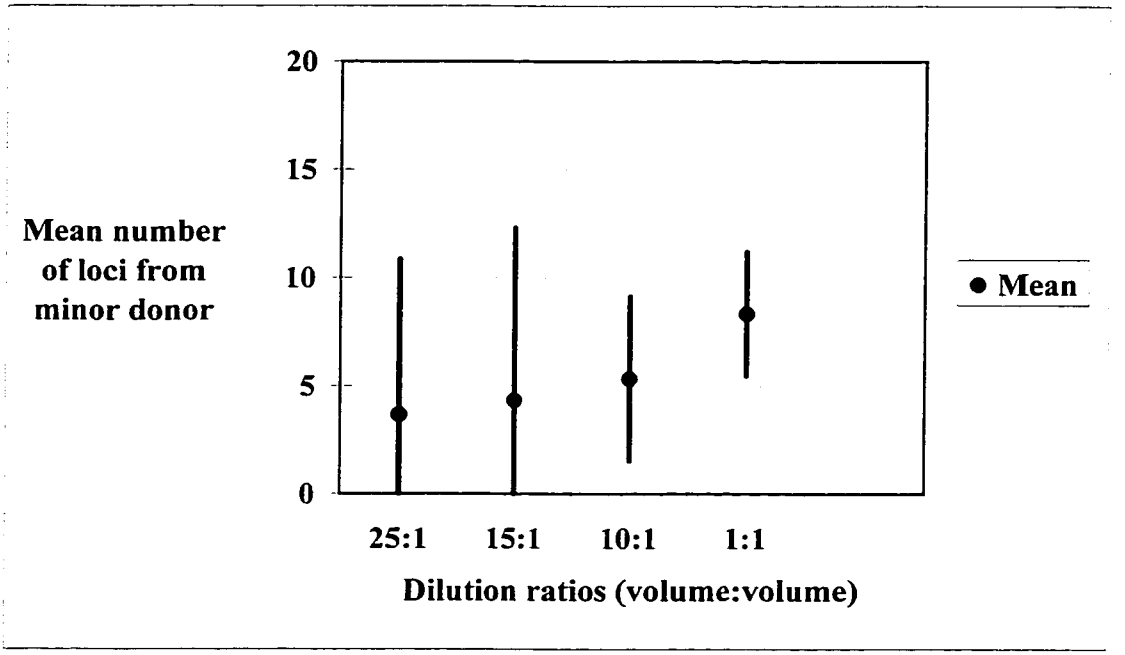

Figure 4a. Epithelial cell fraction of blood/semen mixture when female (blood) is major contributor and male (semen) is minor contributor.
This graph show the mean number of loci and the 95% confidence interval.

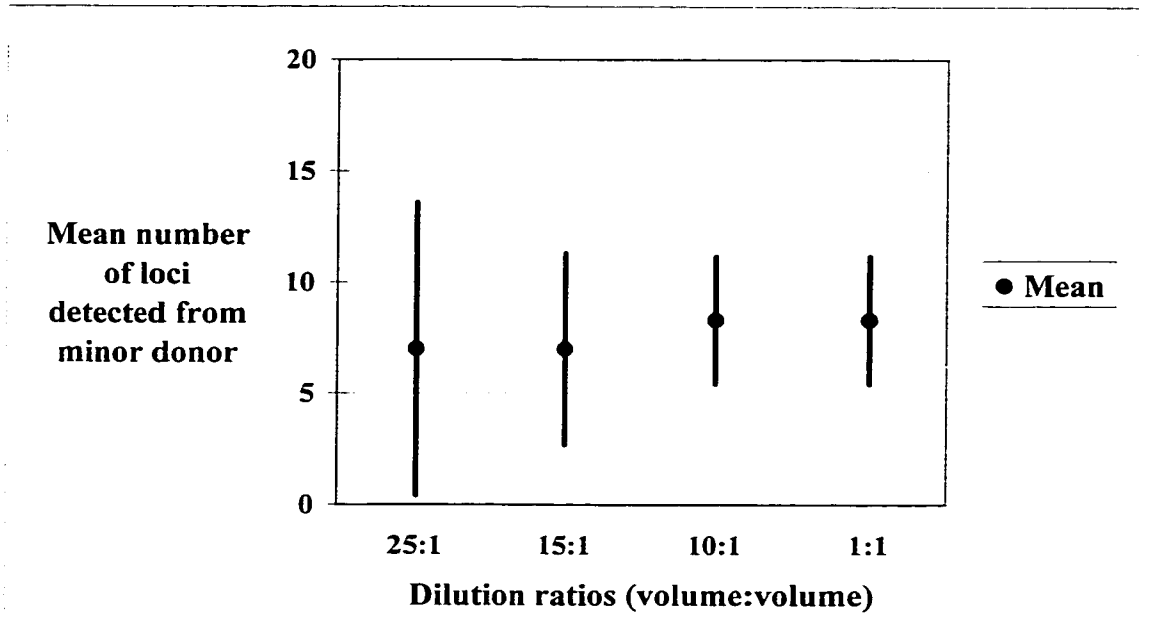

Figure 4b. Epithelial cell fraction of semen/blood mixture when male (semen) is major contributor and female (blood) is minor contributor to the mixture. This graph shows the mean number of loci and the 95% confidence interval.

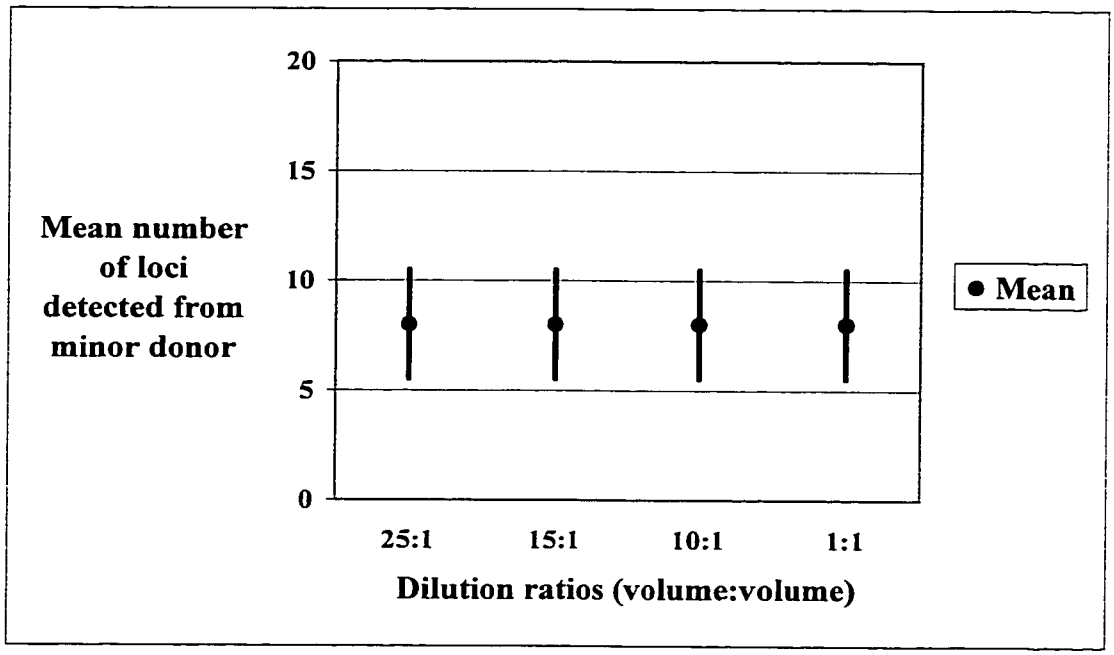

Figure 5a. Sperm fraction of blood/semen mixture when female (blood) is major contributor and male (semen) is minor contributor.
This graph shows the mean number of loci and the 95% confidence interval.

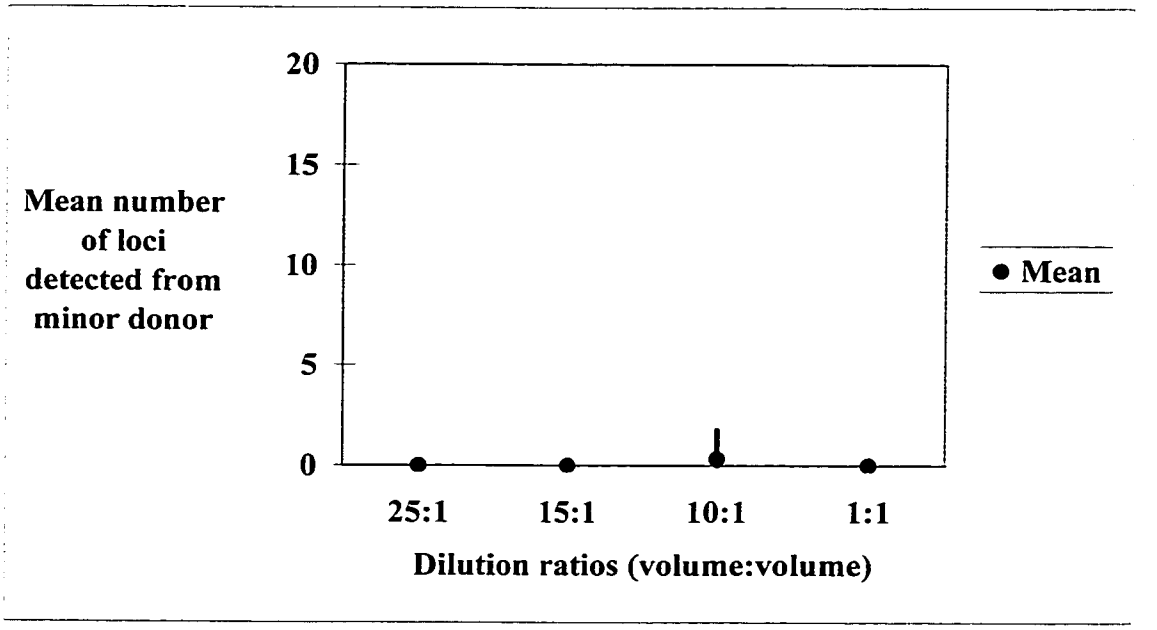

Figure 5b. Sperm fraction of semen/blood mixture when male (semen) is major contributor and female (blood) is minor contributor.
This graph shows the mean number of loci and the 95% confidence interval.

Table 2a, 2b, and 2c. Limits of detection as defined by Wallin et al. (9). The last ratio at which a sample shows evidence of a mixture.

Table 2a. Upper and lower limits of detection for semen/semen mixtures.

Sample #	Upper ratio	Lower ratio
1	15:1	1:15
2	15:1	1:15
3	25:1	1:1
4	1:1	1:25
5	25:1	1:25
6	25:1	1:25

Table 2b. Upper and lower limits of detection for blood/semen mixtures. Eight sperm cell fraction samples showed no evidence of a mixture. The profile for these samples was consistent with that of the semen donor.

Epithelial cell fraction sample #	Upper ratio	Lower ratio	Sperm cell fraction sample #	Upper ratio	Lower ratio
1	25:1	1:25	1	na	na
2	25:1	1:25	2	na	na
3	25:1	1:25	3	25:1	1:10
4	25:1	1:25	4	10:1	1:25
5	25:1	1:25	5	na	na
6	25:1	1:25	6	na	na

na = not applicable

Table 2c. Upper and lower limits of detection for saliva/semen mixtures. Four sperm cell fractions samples showed no evidence of a mixture. The profile for these samples was consistent with that of the semen donor. One 25:1 saliva/semen sperm fraction did not yield any results.

Epithelial cell fraction sample #	Upper ratio	Lower ratio	Sperm cell fraction sample #	Upper ratio	Lower ratio
1	25:1	1:15	1	25:1	1:1
2	15:1	1:25	2	1:1	1:25
3	25:1	1:25	3	25:1	1:25
4	25:1	1:25	4	25:1	1:25
5	25:1	1:15	5*	na	na
6	15:1	1:25	6*	na	na

na = not applicable * = no results obtained for 25:1 sperm fraction ratio

Table 3. The mean DNA concentration (ng/μL) in 25μL of liquid sample.

Liquid sample type	Mean (ng/μL)
Semen (n=12)	128.25
Blood (n=3)	1.88
Saliva (n=3)	1.16

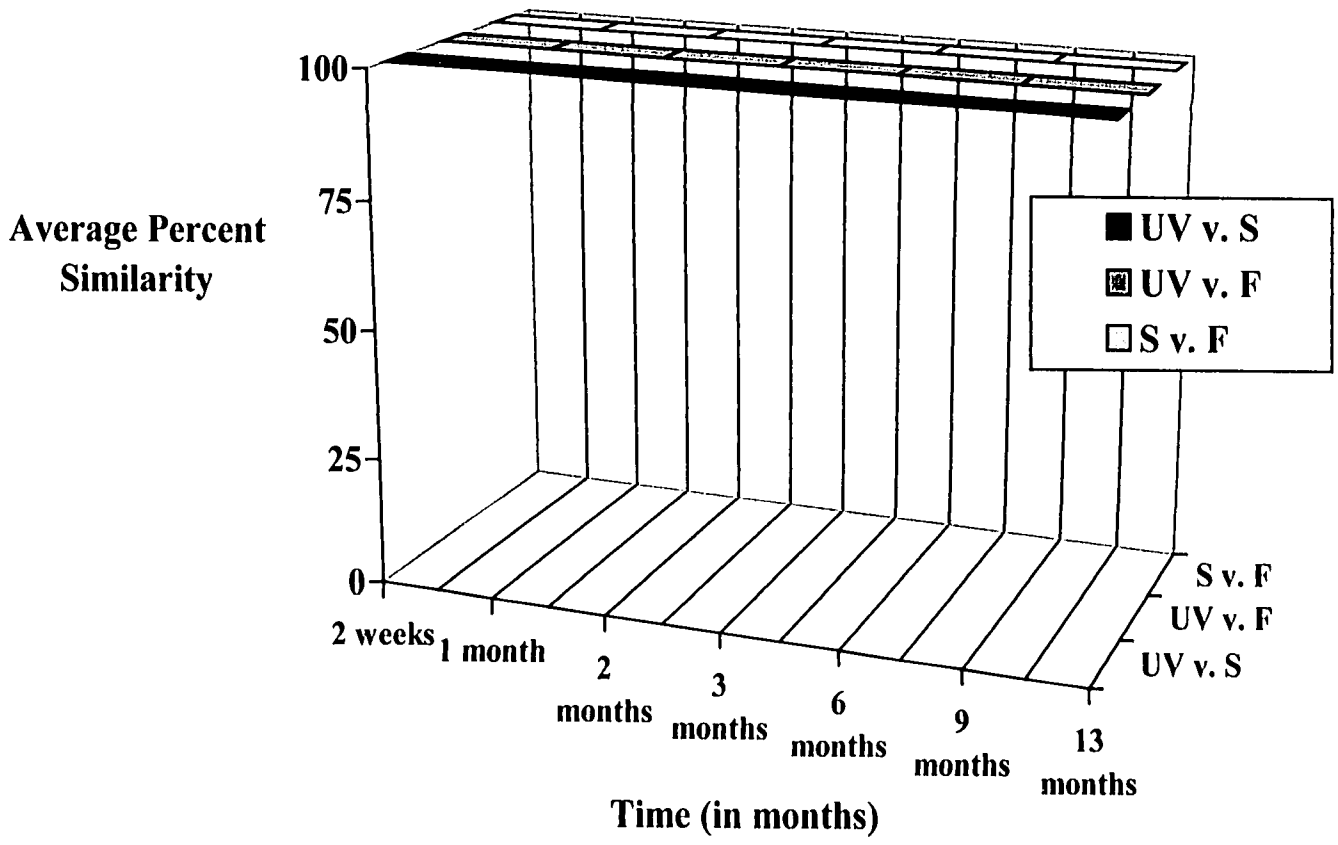

Figure 6. Similarity index for degraded blood samples. The blood samples were unaffected by the three light sources.

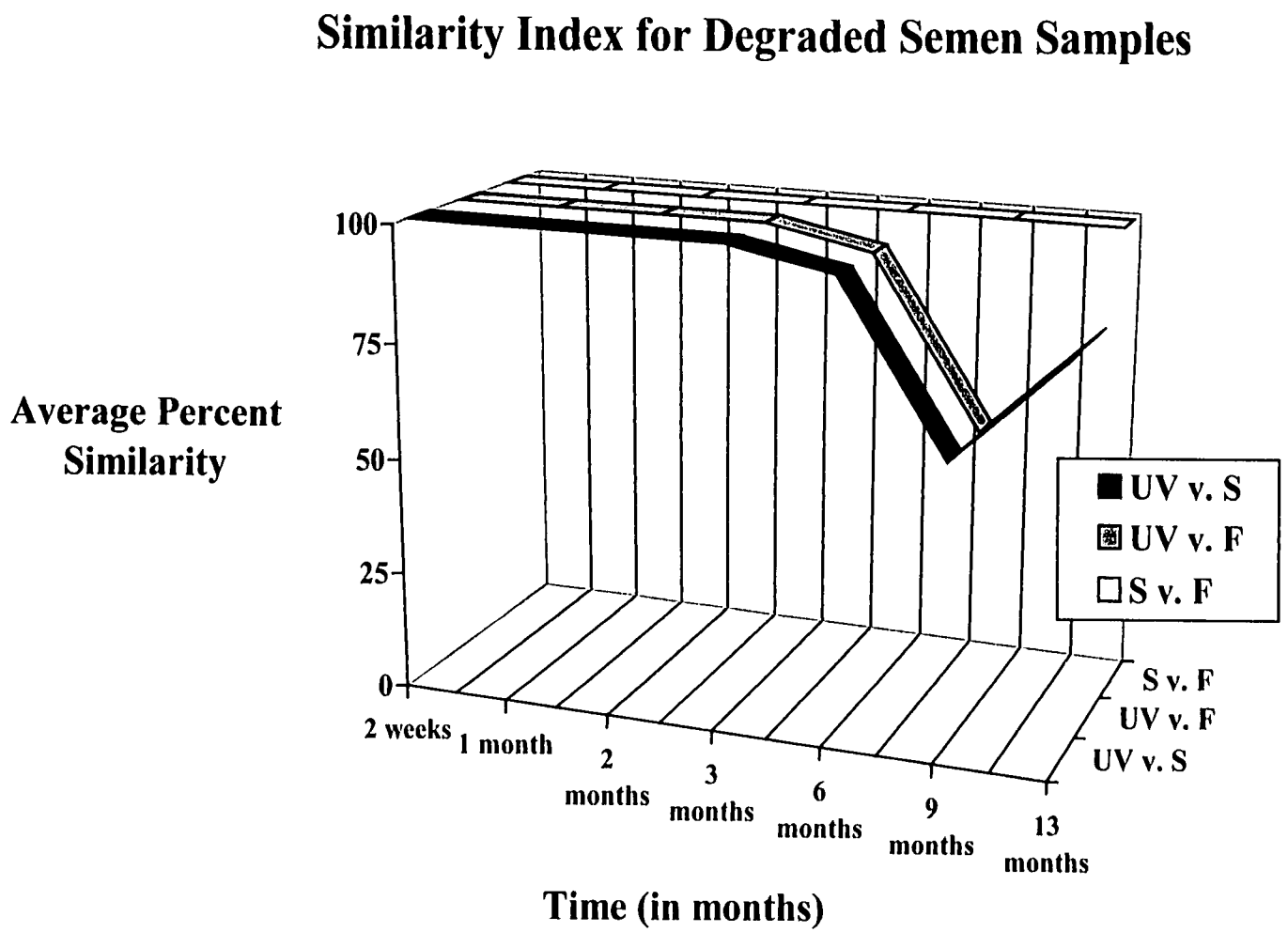

Figure 7. Similarity index for degraded semen samples. Ultraviolet light had some degradative effects on some samples.

Table 4. STR DNA typing results from three semen samples affected by preferential amplification. The reference sample indicates the known genetic profile for the individual that provided the semen samples.

Time interval	D3S1358	VWA	FGA	D8S1179	D21S11	D18S51	D5S818	D13S317	D7S820
Reference sample	16,16	18,19	25,26	11,13	30,31.2	15,20	12,13	10,11	8,10
6 mo.	16,16	18,19	25,26	11,13	30,31.2	---	12,13	10,11	10
9 mo.	16,16	18,19	---	11,13	30,31.2	15	12,13	10,11	---
13 mo.	16,16	18,19	25,26	11,13	30,31.2	15	12,13	10,11	10

--- = no results obtained

Appendix

Digest Buffer: 10mM Tris-HCl – 10mM EDTA – 50mM NaCl – 2% SDS, pH 7.5 (100 mL)

Mix together 1 mL of 1 M Tris-HCl, pH 7.5, 2 mL of 0.5M EDTA, 1 mL of 5M NaCl, 10 mL of 20% SDS (w/v) and 86 mL glass-distilled or deionized water. Store at room temperature.

Phosphate buffered saline (PBS): 2.7mM KCl – 137mM NaCl – 1.5 mM KH_2PO_4 – 8.0 mM Na_2HPO_4, pH 7.4 (1L)

Dissolve 0.2g KCl, 8.0g NaCl, 0.2g KH_2PO_4 and 2.2g Na_2HPO_4-$7H_2O$ in glass distilled or deionized water. Adjust pH of solution to 7.4 if necessary. Adjust to final volume of 1 liter using glass-distilled or deionized water. Sterilize by autoclaving. Store at room temperature.

TE buffer: 10mM Tris-HCl – 0.1mM EDTA, pH 8.0 (1L)

Mix together 10 mL of 1 M Tris-HCl, pH 8.0, 0.2 mL of 0.5M EDTA and 990 mL glass-distilled or deionized water. Sterilize by autoclaving. Store at room temperature.

TBE buffer: 0.45M Tris Borate – 0.01M EDTA (1L) 5X concentrated stock

Dissolve 54g Tris base and 27.5g boric acid in 800mL glass distilled or deionized water. Add 20 mL 0.5M EDTA (pH 8.0). Adjust the volume to 1L with glass distilled or deionized water. The pH range should be 8.0 to 8.3. If pH is greater than 8.3, adjust with boric acid. Dilute concentrated 5X stock 1:10 for a working solution concentration of 0.5X.

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FROM: Nabil Ibrahim,
AVP, Graduate Studies & Research

DATE: March 27, 2000

The Human Subjects-Institutional Review Board has approved your request to use human subjects in the study entitled:

"Biological Fluids: Examination of the Effects of Three Types of Light and Determination of Ratios at which Aminor Component may be Detected"

This approval is contingent upon the subjects participating in your research project being appropriately protected from risk. This includes the protection of the anonymity of the subjects' identity when they participate in your research project, and with regard to any and all data that may be collected from the subjects. The Board's approval includes continued monitoring of your research by the Board to assure that the subjects are being adequately and properly protected from such risks. If at any time a subject becomes injured or complains of injury, you must notify Nabil Ibrahim, Ph.D., immediately. Injury includes but is not limited to bodily harm, psychological trauma and release of potentially damaging personal information.

Please also be advised that all subjects need to be fully informed and aware that their participation in your research project is voluntary, and that he or she may withdraw from the project at any time. Further, a subject's participation, refusal to participate, or withdrawal will not affect any services the subject is receiving or will receive at the institution in which the research is being conducted.

If you have any questions, please contact me at (408) 924-2480.

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